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Award Number: DAMD17-01-1-0382

TITLE: The Role of Fps in Tumor-Associated Angiogenesis

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REPORT DATE: July 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2002	Annual Summary (1 Jul 01 - 30 Jun 02)

4. TITLE AND SUBTITLE
The Role of Fps in Tumor-Associated Angiogenesis

5. FUNDING NUMBERS
DAMD17-01-1-0382

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information).*
Given the poor efficacy of breast cancer treatments, anti-angiogenesis-based approaches represent a promising new alternative for breast cancer treatment. The tyrosine kinase Fps has been implicated in angiogenesis. Expression of activated Fps causes hyper-vascularity in mice (fps^{MF} mice) suggesting that Fps may regulate angiogenesis. Immortalized endothelial cells (EC) have been isolated from these mice suggesting that Fps may promote EC survival. Studies with this cell type have revealed that Fps is activated downstream of platelet derived growth factor which is essential for vasculogenesis. Several other cell types participate in the regulation of angiogenesis including macrophages and platelets. These cells express high levels of Fps and may function abnormally in fps^{MF} mice. In this respect, we have observed increased macrophages counts in peripheral blood and increased populations of abnormally large platelets. We have also observed compromised platelet aggregation suggesting that Fps may modulate coagulation, a process that is tightly coupled to angiogenesis. Using a mouse mammary tumor model we have also shown early tumor onset in the context of loss-of-function Fps genetic backgrounds suggesting that Fps may behave as a tumor suppressor. Thus, Fps may be a suitable target for the development of anti-tumorigenic and anti-angiogenic therapeutics for breast cancer.

14. SUBJECT TERMS
transgenic mice, breast cancer, tumorigenesis, angiogenesis, cytokines tyrosine kinase, oncogenes, hemangioma, metastases

15. NUMBER OF PAGES
8

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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TRAINING REPORT: ANNUAL SUMMARY

Introduction

Angiogenesis plays a critical role in the development of many types of tumors, including mammary tumorigenesis [reviewed in (6, 7)]. Transgenic expression of an activated form of the cytoplamic tyrosine kinase Fps gave rise to mice with pronounced hyperplasia implicating this kinase in angiogenic mechanisms (3). Our work to date has focused on numerous aspects of the angiogenic mechanism that may potentially involve Fps which has led to several developments along this line of investigation. Many of these developments specifically address Objectives in the Statement of Work which were designed to investigate the nature of the role of Fps in angiogenesis. However, other developments have also arisen that were not originally anticipated. These developments have further illuminated how Fps may regulate angiogenic mechanisms. In addition to angiogenesis, Fps has also been implicated in coagulation, immunity, and inflammation [reviewed in (5)], all of which are highly inter-related and are very relevant to the process of tumorigenesis. Thus, Fps may play an important role in modulating tumorigenesis not only through angiogenesis, but through these other processes as well. Understanding how Fps may fulfill this role will be crucial for developing specific therapeutics designed to combat breast cancer.

Progress associated with Statement of Work

Objective #1: Assessment of the effect of *fps* and *fer* allelic variant on tumorigenesis in a mouse breast tumor model.

- **a.** We have successfully generated breeding pairs designed to give offspring that will generate tumors in fps^{0}, fps^{KR} and fps^{MF} genetic backgrounds. Generation of breeding pairs which give rise to tumorigenic fps^{KR}/fps^{DR} and fer^{DR} mice has been delayed due to time constraints and resources.
- **b.** Tumor diameter is currently being monitored in mice with fps^{0} and fps^{KR} genetic backgrounds and is nearing completion. Contrary to what we expected, there is an early onset of tumorigenesis in the context of fps^{0} and fps^{KR} genetic backgrounds. There have been problems generating sufficient number of tumorigenic mice with a fps^{MF} genetic background. It is suspected that embryos of this genotype are dying *in utero*; this may be expected since there is some evidence for decreased viability of offspring from hemizygous fps^{MF} x *wild-type* (*wt*) breeding pairs.
- **c.** Lung metastases is currently being monitored in tumorigenic mice harboring fps^{0} and fps^{KR} alleles.
- **d.** We have quantified the vascular area in fps^{MF} mice using intra-vital microscopy on cremaster muscle. Our results indicate that there is a 1.7-fold increase in vascular area in these mice. The analysis also revealed that the vascular system is highly disorganized and tortuous in nature.

Objective #2: Assessment of the endothelial cell (EC) immortalizing ability of *fps* relative to the known EC immortalizing/transforming agent polyoma middle T(PymT).

- **a.** pMSCV-based retroviruses encoding PymT, *fps*, fps^{KR} and fps^{MF} have been successfully generated for the purposes of transducing primary cells derived from day 12 yolk-sacs.

b. Primary cells from day 12 yolk-sacs of *wt* mice were transduced with the retroviruses generated in Objective #2a and transduced cells were isolated using puromycin drug selection. Preliminary attempts to quantify EC composition failed either because the EC marker employed was not ideal, or because the level of ECs in the selected cells was extremely low. Work on this objective is ongoing, although priority has temporarily been lowered in favor of other objectives.

Objective #3: Generation of EC lines from different *fps* genetic backgrounds for *in vitro* studies.

a. pMSCV-based retroviruses encoding PymT and SV40-T have been successfully generated.

b. Several attempts have been made at generating endothelial cells from aortic and lung explants. These attempts were complicated by the known difficulty and inconsistency of these methods. In most cases the cell cultures generated from the explants were highly heterogeneous. Cultures appeared largely fibroblast-like after transduction and selection with PymT. Subsequent viable cell-sorting using EC-specific markers has proven to be very difficult. We are currently assessing the possibility of employing magnetic cell sorting to isolate EC cells for the purposes of generating EC lines.

Objective #4: Assessment of the role of Fps in angiogenesis *in vitro*.

a. Experiments preparing mouse fibrin-immobilized aorta and yolk-sac from *wt* and fps^{MF} genetic backgrounds have been performed. We have successfully been able to observe neovascularization into the surrounding fibrin matrix using these types of organ explants and are currently assessing neovascularizton in abdominal muscle fragments.

b. Preliminary experiments have not indicated any significant differences in neo-vascularization between *wt* and fps^{MF} mice in yolk-sac explants. These results are not conclusive however, because we have been unable to observe consistent neovascularization even within the same genotype. We are currently optimizing this protocol.

c. Assessment of angiogenesis using perforated polycarbonate chambers implanted in mice has not been performed, as we are awaiting the results from Objective 4b.

d. Completion of Objective 3b is required for testing the ability of immortalized ECs to form tubules in fibrin-gels.

e. C166 cell lines expressing myc-epitope-tagged forms of kinase-inactive Fps (FpsKR), myristoylated Fps (MFps) and Fps have been generated.

f. We have tested the ability of cell lines generated in Objective 4e to form tubules in fibrin gels. Preliminary results have not demonstrated any differences in the tubule-formation capacity of these cell lines.

Objective #5: Assessment of coagulation and fibrinolytic parameters in fps^{MF} mice.

a. Standardized PT and APTT assay have been scheduled to be completed in months 25-30. However, we have determined coagulation parameters using platelet aggregation and tail bleeding assays (see key accomplishments below).

b. *In vitro* clot lysis assays have been scheduled to be completed in months 31-36.

Objective #6: Examination of proteolytic expression profiles of cell lines expressing Fps.

a. Matrix metalloproteinase expression profiles of primary macrophages have been examined. We have observed enhanced gelatinase activity of a band migrating at 90 kDa in fps^{MF} mice. The identity of this band is unknown, although based on migration characteristics it may represent MMP9 which has been heavily implicated in proangiogenic function.

b. Examination of uPA and MMP-2/-9 activity of cell cultures of immortalized ECs will be completed pending achievement of Objective #3. However, uPA and MMP-2/-9 expression levels of the cell lines generated in Objective 4e are currently being tested.

Objective #7: Elucidation of the role of Fps in signaling pathways potentiated by VEGF, bFGF and thrombin.

a. We are continuing our study on thrombin signaling in C166 cells. We initially observed that Fps/myristoylated Fps (MFps) is activated downstream of the thrombin receptor in C166 cells. Our data to date suggest that the activation of Fps/MFps is a late event and appears to be dependent on cell density. Delayed Fps/MFps activation suggests that these proteins are phosphorylated in response to an autocrine system triggered by thrombin stimulation. We are currently delineating the mechanics of this response. Preliminary studies have shown that C166 cells are insensitive to basic-fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), however, we have observed rapid dose-dependent activation of these cells to platelet-derived growth factor (PDGF), a factor known to be essential for vasculogenesis.

b. bFGF, VEGF, and thrombin signaling in immortalized ECs in the different Fps genetic backgrounds will begin upon completion of Objective #3.

Key accomplishments related to Fps and its role in angiogenesis.

- Early onset of tumorigenesis has been observed in fps^{0} and fps^{KR} genetic backgrounds. There was no change, however, in the rate of tumorigenesis. Early onset is contrary to our expectations and suggests that Fps may play a role as a tumor suppressor.

- Using intra-vital microscopy of cremaster muscle, we have determined that there is a 1.7-fold increase in total blood vessel area of fps^{MF} mice. We also report that the vascular system is highly disorganized and tortuous in nature.

- MFps is activated in immortalized endothelial C166 cells downstream of PDGF, a growth factor that has been implicated in vascular remodeling.

- Understanding the general physiological phenotype of fps^{MF} mice will be essential for uncovering the basis of the proangiogenic phenotype of these mice. To date, we have observed, splenomegaly, cardiomegaly, and decreased steady-state levels of blood pressure in fps^{MF} mice. In addition, there may be defects in vascular integrity, as suggested by experiments showing compromised histamine-induced vascular permeability.

- Macrophages are important modulators of angiogenesis. Fps has been highly implicated in myelopoiesis, the process which gives rise to granulocytes, and monocytes. Macrophages are activated monocytes and Fps has been shown to be expressed at high levels in both immature myeloid cells and in monocytes/macrophages [reviewed in (5)]. We therefore hypothesized that Fps may modulate angiogenesis through an effect on macrophages. Hematological analysis has revealed increased output of cells of the myeloid lineage, and bone-marrow and spleen flow-analysis has suggested some minimal perturbations in myelopoiesis. In addition, GM-CSF signaling may be altered in bone-marrow macrophages islolated from these mice. Taken together, these results suggest that myelopoiesis may be perturbed to some extent in *fps*^{MF} mice.

- Hematological analysis has also revealed decreased output of platelets and RBCs. This output is coupled with observations of increased erythroid progenitor levels in *fps*^{MF} mice. Pathological examination of these mice using electron- and light-microscopy has shown that platelet populations of increased size exist. There also exists a heterogeneous array of red blood cell structural defects including acanthocytosis. These data provide additional evidence of perturbed myelopoiesis in these mice. Moreover, the existence of RBC and platelet defects suggests that coagulation may be perturbed in these mice. Indeed, we have observed compromised platelet aggregation and bleeding defects in these mice and we will be further investigating coagulation function in Objective #5. Reports describing defects in platelet aggregation have been submitted for publication (See below). Interestingly, platelets play important roles in the highly inter-related processes of coagulation and angiogenesis (2). Thus, it is possible that certain aspects of the mechanism of angiogenesis in these mice may be indirectly affected as a result of coagulation defects arising from compromised platelet aggregation or from other coagulation-associated defects.

Reportable Outcomes

Abstracts

W. Sangrar, R.A. Zirngibl, J. Mewburn, Y.A. Senis, C. Chapler, D.H.Lee and P.A. Greer. A murine transgenic line expressing a myristylated form of Fps/Fes is characterized by disorganized hyper-vascular patterning and peripheral blood defects. *Blood* **98**, 31a. *43rd Annual A.S.H. Meeting*. Orlando, Florida, 7th-12th December, 2001.

Manuscripts *submitted* for publication June 2002

Y.A. Senis, **W. Sangrar**, R.A. Zirngibl, A.W.B. Craig, D.H. Lee, and P.A. Greer. Fps/Fes and Fer Nonreceptor Protein-Tyrosine Kinases Regulate Collagen- and ADP-Induced Platelet Aggregation. Submitted to *Journal of Cell Biology*, June 2002

W. Sangrar, Y.A. Senis, M. Richardson, and Peter A. Greer. Transgenic Mice Expressing an Activated Mutant Fps/Fes Nonreceptor Protein-Tyrosine kinase are Characterized by Bleeding Defects, Thrombocytopenia and Increased Platelet Volume, Submitted to *Journal of Cell Biology*, June 2002.

Conclusions

In genetic backgrounds in which there is a loss-of-Fps-function (*fps*^{0} and *fps*^{KR} genetic backgrounds) we have observed an increased onset of tumorigenesis. Angiogenesis is a known critical step in tumorigenesis and therefore we had expected a late onset based on the hypothesis that loss-of-function mice maybe hypo-angiogenic. This hypothesis is derived from knowledge of the proangiogenic phenotype of *fps*^{MF} mice. The results reported here, are nonetheless intriguing since they suggest a role for Fps in tumorigenesis as a potential tumor suppressor. Thus, it will be important to address the how Fps may behave as a suppressor of breast tumorigenesis. It will also be important to address, whether gain-of-function Fps variants (*fps*^{MF} mice) are associated with delayed tumorigenesis. If so, it could provide the rational for the design of therapeutics that mimic the tumor-suppressor-like effects of Fps on tumorigenesis

The presence of a proangiogenic phenotype in *fps*^{MF} mice underscores an important role for this kinase in regulating mechanisms of angiogenesis. Understanding the nature of this role is important in order to provide the rational for the design of anti-angiogenesis inhibitors for the treatment of breast and other cancers. Fps is highly expressed in macrophages(4), platelets (Senis et al, *submitted for publication*), and ECs (1) and we are examining the role of Fps in these cells. Studies to date have shown that the ontogeny of development of these cell types appears abnormal in *fps*^{MF} mice and there appear to be Fps-associated defects in the function of these cells including defects in platelet aggregation. Since these cell types play very important roles in both of the highly inter-related processes of coagulation and angiogenesis (2), it is likely that Fps may modulate vessel development through effects on ontogeny and function of macrophages, platelets and ECs.

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